# STUDY OF AN ANTHRAX PROTECTIVE ANTIGEN

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ABSTRACT: The antigen is said to be one of three components of an extracellular toxin produced by the anthrax agent. It is produced by incubation in a milk-peptone medium. Data are presented on tests on white mice, guinea pigs, rabbits, sheep and monkeys, indicating it is equal to or better than live STI vaccine.

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A protective antigen of the anthrax microbe was first obtained in vitro by Gladstone, in 1946. The antigens previously separated from the cells of these microbes (somatic--polysaccharides and capsules--polypeptides) did not have the ability to establish immunity against anthrax in inoculated animals. It was later found out that formation of specific nonsusceptibility to anthrax is due only to an extracellular protective antigen. This antigen is the product of the vital activity of B. anthracis, escaping into the surroundings in the process of growth of the microbe in vivo, as well as in vitro.

It was established that the protective antigen is part of a complex, determining the virulence and pathogenicity of the anthrax microbe. The toxin produced by the anthrax agent consists of three components, one of which is the protective antigen. Each of the three components of the toxin has little activity in itself; however, the mixture of them is highly toxic. Therefore, the protective antigen is not only a factor in immunogenicity, but a component part of the virulence factor of the anthrax microbe.

We have conducted research to obtain a protective anthrax antigen in the following directions: a nutrient medium has been developed for cultivation of the microbe, the optimum conditions of biosynthesis of the antigen and methods

of isolation, concentration and purification of it have been studied, and the biological and chemical properties of the latter have been determined, as well.

We have used a milk-peptone medium for cultivation of the microbes; it is more available and cheaper than all described in the literature. The STI-1 strain was used as the antigen producer. A daily culture was seeded, based on 5000 microbe cells per ml of medium. Upon completion of culturing, the microbe cells were removed initially by centrifuging, then by filtering through paper pulp and, finally, by means of sterilizing filtration through a ceramic plug or a glass-orifice #4 filter.

In study of the effect of different components of the milk-peptone medium on growth of anthrax microbes and biosynthesis of the extracellular protective antigen, a significant role of the organic compounds used as the energy source was established. Glucose, saccharose and dextran proved to be the best for this purpose.

The hydrogen ion concentration necessary for the vital activity of the microbes was provided by addition of sodium bicarbonate to the medium.

Study of the dynamics of accumulation of the protective antigen in the culture fluid, during incubation of the anthrax microbes on milk-peptone medium, showed that the antigen appears in the medium in the 10-13th hour of incubation, in the logarithmic growth phase. The immunogenic activity of the culture filtrates then increased continually and, by 20-24 hours of incubation, i.e., by the end of the stationary phase, it reached a maximum. Further incubation led to a reduction in the immunogenic activity of the culture fluid.

As a result of selection of the nutrient medium and study of the conditions of antigen formation by growth on this medium, the conditions for culturing

anthrax bacteria, for the purpose of producing the protective antigen, was worked out under both laboratory conditions and experimental production conditions.

To produce the antigen under laboratory conditions, anthrax microbes were cultured in 5-liter bottles, containing 1 l of milk-peptone medium, for a period of 20-24 hours. In experimental production, 100 l cultivators, into which 50 l of medium was poured, were used for this purpose. Incubation lasted 20-24 hours (without additional aeration).

For isolation, concentration and purification of the protective antigen, a number of methods, normally used for isolation of protein preparations, were used.

We present material in this report, concerning the immunogenic activity of the protective antigen, produced by sorption of the native antigen on aluminum oxide hydrate gel.

In study of the sorption conditions of the protective antigen, as a function of pH and amount of sorbent, it turned out that preparations sorbed by addition of 0.8% aluminum hydroxide to the culture fluid at pH 7.0 were the most effective. The $ED_{50}$ for white mice was 0.017 ml. 0.4% formalin was added to the sorbed antigen, produced by the method described, as a preservative.

The immunological activity of the sorbed protective antigen was studied in white mice, guinea pigs, rabbits, sheep and monkeys.

Mice weighing 15-17 g were immunized subcutaneously, once and twice, with sorbed antigen, at doses of 0.5 – 0.1 or 0.02 ml. In the double immunization, the interval between injections was 7 days. Immunity was tested 14 days after

completion of immunization, by means of intraperitoneal infection with a 2-day culture of STI vaccine (vaccine for cutaneous inoculation of people, produced by the Tbilisi Institute of Vaccines and Serums), in the amount of 2-5 billion (SDc1).

Single and double administration of the sorbed antigen to white mice ensured their protection from the intraperitoneal infection with a dose of 5 Dc1 (Table 1). However, the level of protection was higher with the double immunization than with the single.

Table 1. Immunogenicity of Sorbed Antigen for Mice

| a | b | | c | d |
|---|---|---|---|---|
| | e | f | | |
| g | 0,02 | 0,2 | 1:10 | |
| | 0,1 | 1 | 7:10 | 0,77 |
| | 0,5 | | 9:9 | |
| h | 0,02 | 0,2 | 0:10 | |
| | 0,1 | 1 | 3:8 | 1,86 |
| | 0,5 | 5 | 5:10 | |
| i | | | 0:10 | |

**[Translator's Note: Commas in tabulated numerals are equivalent to decimal points.]**

Key: a--immunization of animals
b--vaccine dose
c--result
d--$ED_{50}$ (in mg)
e--ml
f--mg
g--twice, 7-day interval between injections
h--once
i--control (non-immunized animals)

Designations: numerator, number of mice surviving; denominator, number of mice tested.

In tests with guinea pigs (weighing 250-300 g), the immunogenicity of the sorbed antigen was studied by various immunization schemes. The results of single, double and triple injections of the antigen were tested. In the

twofold immunization, the preparation was administered at 14- and 30-day intervals and in the triple, at 7-day intervals (Table 2). 14 days after the last injection, the guinea pigs were infected subcutaneously with a 2-day culture of the second vaccine of Tsenkovskiy, in the amount of 100 million microbe cells (10 Dcl).

Table 2. Immunogenicity of Sorbed Antigen for Guinea Pigs

| a | b | c | | d | e | |
|---|---|---|---|---|---|---|
| | | f | g | | | |
| h | — | 2 | 20 | 1/5 | 20 | 20 - 17,9 |
| i | 40 | 0,5 | | 5/7 | 66 | 66 - 16,7 |
| | | 2 | 20 | 7/10 | 70 | 70 - 17,29 |
| i | 14 | 0,5 | 5 | 3/10 | 30 | 30 - 17,56 |
| | | 2 | 20 | 4/10 | 40 | 40 - 16,31 |
| j | | 0 | | 8/10 | 80 | 80 - 16,1 |
| | | 2 | 20 | 7/10 | 70 | 70 - 13,1 |
| k | - | - | — | 0/5 | 0 | |

Key: a--immunization of animals
b--interval between injections (in days)
c--antigen dose
d--result
e--% surviving
f--ml
g--mg
h--single
i--double
j--triple
k--control (non-immunized animals)

Designations: numerator, number surviving; denominator, number of animals infected.

The double immunization with a 14-day interval and the triple one provided identical results. Survival in these groups of animals, regardless of immunizing dose, was 20-30%. With increase in the interval between injections to 30 days, the resistance of the animals increased sharply (to 70% and higher).

The high immunogenic activity of the sorbed antigen was also found in acute tests on rabbits. The animals were immunized with various doses of sorbed antigen, 10, 25, 50, 100 mg, subcutaneously, twice, with a 38-day interval between injections. 20 days after the last injection, the animals were infected, by administration of 5000 spores of anthrax strain #836 (100 Dlm).

Immunization with sorbed antigen, at a dose of 10 mg, provided protection to the greater part of the injected animals from infection with the highly virulent anthrax culture (Table 3).

Table 3. Immunogenicity of Sorbed Antigens for Rabbits

| a | b | c | d | e | |
|---|---|---|---|---|---|
| Иммунизация животных | Доза антигена (в мг) | Интервал до заражения (в днях) | Доза заражения | Результат | |
| f | | | | | |
| [illegible] | 100 | 20 | 5000 | 9/9 | 100 |
| [illegible] | 50 | 20 | g | 9/9 | 100 |
| [illegible] | 25 | 20 | [illegible] | 5/10 | 100 |
| | 10 | 20 | N 836 (100 Dlm) | 8/10 | 80 |
| h | | | | | |
| [illegible] | — | | | 0/12 | |

Key: a--immunization of animals
b--antigen dose (in mg)
c--interval before infection (in days)
d--dose of infection
e--result
f--twice, subcutaneous, with 38-day interval
g--5000 spores of strain #836 (100 Dlm)
h--control (non-immunized animals)

Designations: numerator, number surviving; denominator, number of animals infected.

The immunogenicity of the sorbed preparation of protective antigen also was tested on sheep (Table 4). There were 100 Russian and Romanov stock sheep in the test: 60 sheep were innoculated with sorbed protective antigen

subcutaneously, twice, with an interval of 19 days, in which 20 of them received 75, 20 - 150 and 20 - 300 mg of the antigen in the immunization period; 30 sheep were immunized once, with 150 mg of the sorbed antigen administered to 10 sheep and 20 million spores of live STI vaccine to 20; 10 animals were left as controls.

Table 4. Immunogenicity of Sorbed Antigen for Sheep

| a | b | | c | d |
|---|---|---|---|---|
| | e | f | | |
| g | 75 (25, 50) | 2 | 10 | 9 10 |
| | | | 100 | 10 10 |
| | 150 (50, 100) | 4 | 10 | 10 10 |
| | | | 100 | 10 10 |
| | 300 (100, 200) | 8 | 10 | 10 10 |
| | | | 100 | 10 10 |
| h | 150 | 4 | 100 | 5 5 |
| | | | 100 | |
| i | 25 × 10⁶ | 0.2 | 100 | 9 10 |
| | | | 100 | 9 10 |
| k | | | 10 | 0 2 |
| | | | 10 | 0 2 |
| | | | 100 | 0 2 |

Key: a--immunization method
b--vaccine dose
c--dose of infection (in $10^3$)
d--result
e--mg
f--ml
g--native sorbed antigen subcutaneously, twice
h--native sorbed antigen subcutaneously, once
i--STI vaccine
j--$25 \times 10^6$
k--non-immunized animals

Designations: numerator, number surviving; denominator, number of animals infected.

25 days after injection, the sheep were infected. Half of the sheep of each group were administered 10,000 spores of anthrax strain #836 (10 Dcl) and the other half, 100,000 (100 Dcl). On the seventh day after the 10 Dcl

infection, one of the sheep, inoculated twice with protective antigen, at a dose of 75 mg, died. But the remaining sheep, injected both once and twice, survived. Two sheep died in the group of animals immunized with live STI vaccine.

The data obtained demonstrated that an intense immunity against anthrax was observed after almost a month, in sheep immunized once and twice with sorbed protective antigen and in those injected with STI vaccine.

Similar data were obtained in tests on monkeys (Table 5). There were 21 monkeys in the test, of which 12 underwent subcutaneous immunization with sorbed protective antigen, 6 were injected with live STI vaccine and 3 remained for the control. 14 days after the injections, all monkeys were infected intracutaneously with various doses of highly virulent anthrax strain #836.

Table 5. Study of Immunogenic Effectiveness of Sorbed Antigen in Acute Tests on Monkeys with Subcutaneous Administration

| a | b | c | d |
|---|---|---|---|
| e | | 1 | |
| | | | 2 3 |
| f | | | |
| | | 1 | |
| | | 5 | 3 3 |
| g | h | 1 | 3 3 |
| | | 5 | 1 3 |
| i | | 1 | 0 1 |
| | | 5 | 0 2 |

Key: a--immunization method
b--antigen dose (in mg)
c--dose of infection (number of spores, in thousands)
d--result
e--twice, with sorbed protective antigen
f--three times, with sorbed protective antigen
g--with STI vaccine
h--50 million spores
i--unvaccinated (control)

Designations: numerator, number surviving; denominator, numer of animals infected

Upon infection with a dose of 1000 spores, all monkeys injected with both the sorbed protective antigen and the live STI vaccine survived. Upon infection with 5000 spores, 1 of 3 immunized twice died and, of 3 injected with live STI vaccine, 2 monkeys died. All the animals immunized 3 times survived.

The results of the tests on monkeys again confirmed the marked immunogenic activity of the protective antigen, sorbed on aluminum oxide hydrate.

Testing of protective anthrax antigen in immunization tests of various types of animals, in confirmation of data in the literature, results in the conclusion that it is possible to use it, for purposes of active prophylaxis of anthrax. The protective antigen of anthrax microbes can be recommended for use as a chemical anthrax vaccine

## CONCLUSIONS

1. Anthrax microbes cultured in vivo discharge an extracellular protective antigen to the surrounding medium, which causes formation of specific immunity.

2. To incubate anthrax microbes, for the purpose of producing the protective antigen, a nutrient medium is proposed, the base of which is milk and peptone.

3. In tests of a number of methods of isolation of the protective antigen from culture filtrates, sorption by aluminum oxide hydrate proved to be the best.

4. A regulation has been worked out for production of the extracellular protective anthrax antigen under laboratory conditions, and the possibility has been demonstrated of producing the antigen under semi-production conditions.

5. High immunogenic activity of the sorbed preparations of protective antigen has been disclosed, in acute tests on white mice, guinea pigs, rabbits, sheep and monkeys.

6. The high immunogenicity of the sorbed protective antigen for animals is a basis for concluding that this preparation can be used as a chemical vaccine against anthrax.